Metabolic Pathways Affecting Toxicity of *N*-Acetyl Zectran

Raymond P. Miskus, Theresa L. Andrews, and Melvin Look

N-Acetylation of the insecticide Zectran does not greatly alter its toxic effects on spruce budworm, but virtually eliminates toxicity in mice. The fate of acetylated Zectran was investigated by using several radioactive labels. Mice detoxify acetyl Zectran by hydrolysis of the carbamic acid ester, producing CO_2 and 4-dimethylamino-3,5-xylenol and an unknown water-soluble compound. Spruce budworm remove the acetyl group, regenerating Zectran and metabolizing part of it to 4-methylamino-3,5-xylyl-*N*-methyl carbamate.

Robertson *et al.* (1965) reported that *N*-acetylation of aryl-*N*-methyl carbamates drastically reduced mammalian toxicity. In testing Zectran (4-dimethylamino-3,5-xylyl-*N*-methyl carbamate) on mice, Miskus *et al.* (1968) found that *N*-acetylation of this insecticide had the same dramatic effect of lowering acute oral toxicity. But this decreased toxicity did not appear when Zectran was tested on spruce budworm larvae (*Choristoneura fumiferana* Clemens). [Common and scientific names have been changed to western budworm (*Choristoneura accidentalis* Freeman).] To determine the reasons for this selectivity, the metabolism of *N*-acetylated Zectran in mice and in spruce budworm was studied.

METHODS AND MATERIALS

^{14}C-Zectran radiolabeled in the carbonyl position was purchased from New England Nuclear (Chicago, Ill.), and had a specific activity of 6.3 mc. per mmole. The *N*-acetyl Zectran was ^{14}C-labeled in three positions: the acetyl carbonyl with a specific activity of 2.0 mc. per mmole; the carbamyl carbonyl with an activity of 6.3 mc. per mmole; and the methyl carbons of the 4-dimethylamino position with an activity of 0.6 mc. per mmole.

Carbamylcarbonyl-^{14}C-*N*-acetyl Zectran and (dimethylamino)-^{14}C-*N*-acetyl Zectran were synthesized by procedures adapted from Fraser, Clinch, and Reay (1965). Purity of these compounds was previously established (Look, 1968). The procedure for both syntheses was identical. In a 5-ml. round-bottomed flask, fitted with a condenser and a calcium chloride drying tube, 2.25 μc. of labeled Zectran, 2 ml. of acetic anhydride, and one drop of concentrated sulfuric were heated under reflux for 4 hours. The solution was allowed to cool, diluted with 20 ml. of ice water, and made slightly basic with sodium bicarbonate. The mixture was then extracted with three 20-ml. portions of methylene chloride. The combined extract was dried by passage through anhydrous sodium sulfate, evaporated to a small volume with a warm nitrogen stream, and spotted on a 400-micron-thick silica gel G, 8 × 2 inch plate. Resolution was made with a hexane–ethyl ether (2 to 1) developing solvent.

The *N*-acetyl Zectran spot, detected by its radioactivity, was removed by extraction of the silica gel with methylene chloride. The methylene chloride was removed by evaporation, leaving a residue of labeled *N*-acetyl Zectran. The material was identical to a known sample of nonlabeled *N*-acetyl Zectran as determined by silica gel G thin-layer chromatography using two solvent systems described later.

The synthesis of *N*-acetyl Zectran-(acetyl carbonyl)-^{14}C is far more complex (Look, 1968).

Female mice (25-gram Swiss strain) were orally dosed with 60 mg per kg. (LD_{50} < 1000 mg. per kg.) of the radioactive acetylated Zectran and 10 mg. per kg. (LD_{50} 30 to 50 mg. per kg.) of the radioactive Zectran as described by Miskus *et al.* (1968). The animals were placed quickly in a glass metabolic chamber (Figure 1). Air was drawn through the chamber at 100 ml. per minute. Trap I removed water. Trap II contained 2-methoxyethanol, and was added to prevent trapping of organic radioactive compounds in trap III, which collected $^{14}CO_2$. Trap III contained 2 ml. of ethanolamine and 4 ml. of methoxyethanol (Smith *et al.*, 1964). Samples were collected during the 48-hour experiment.

The metabolic $^{14}CO_2$ and urinary radioactivity were counted in a scintillation cocktail described by Jeffay and Alvarez (1961). The radioactive measurements were made with a Packard Instruments liquid scintillation spectrometer. Quench corrections for the liquid scintillation counting were made with an external standard as described by Hayes *et al.* (1964). After 48 hours, feces were homogenized with methylene chloride and water (1 to 1). The mouse was sacrificed, autopsied, and homogenized with water and ethyl ether. Each resultant homogenate of the feces and mouse was resolved into two liquid layers by centrifugation, and aliquots of the phases were counted for radioactivity, as described.

Sixth instar spruce budworm larvae (average 150-mg. weight) were treated by topical application to the dorsal thorax at a

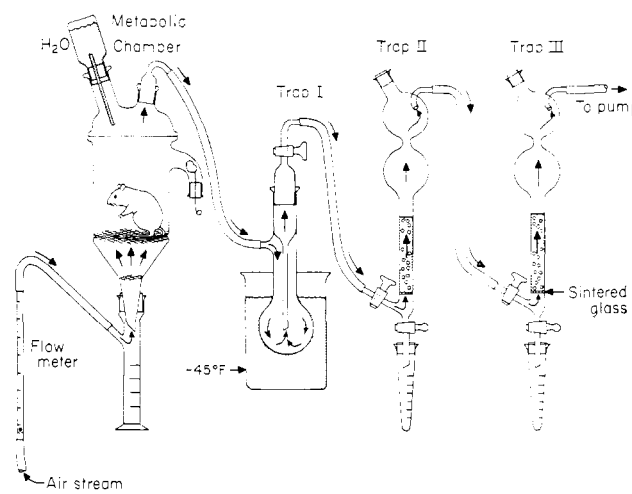

Figure 1. Chamber used to study metabolism of acetylated Zectran

Pacific Southwest Forest and Range Experiment Station, Forest Service, U. S. Department of Agriculture, Berkeley, Calif. 94701

Table I. Recovery of Radioactivity from Treated Mice

Chemical and Position of ^{14}C Label	Hours[a]	Per Cent Recovery CO_2	Urine	Chamber washings	H_2O trap	Feces	Carcass	Total
Carbonyl ^{14}C-Zectran	6[a]	68.6	...	...	...	...	...	...
	48	75.0	16.6	0.5	<0.1	7.0	2.5	101.6
Acetyl carbonyl-^{14}C-*N*-acetyl Zectran	6[a]	1.1	...	...	...	...	...	...
	48	2.5	62.6	5.2	<0.1	24.7	1.4	96.4
Carbamyl carbonyl ^{14}C-*N*-acetyl Zectran	2[a]	57.8	...	...	...	...	...	...
	48	71.7	17.2	7.9	<0.1	0.7	6.7	104.2
Dimethylamino-^{14}C-*N*-acetyl Zectran	6[a]	2.0	...	...	...	...	...	...
	48	2.6	71.7	20.3	<0.1	0.9	0.5	96.0

[a] 2 and 6 hours indicate rapidity of $^{14}CO_2$ recovery.

0.25-μg. dose per insect using acetone as a solvent. These radioactively dosed larvae (25 to 50 per experiment) were placed in glass vials connected to the metabolic chamber used for mice. The air was drawn through at 20 ml. per minute (Metcalf *et al.*, 1967). $^{14}CO_2$ was collected in the same manner as in the test with the mice. Each insect was washed with methylene chloride at the end of the experiment. The insects were homogenized, and the radioactive metabolites were extracted with 0.5*N* sulfuric acid according to the purification procedure described by Pieper and Miskus (1967). Aliquot samples from the $^{14}CO_2$ traps, external washings, and the homogenized insects were counted for total radioactivity using the liquid scintillation spectrometer.

The metabolites found in the mice urine and insect samples were examined quantitatively and qualitatively by spotting silical gel G thin-layer plates. These plates were developed in a solvent system consisting of ethyl ether–hexane (4 to 1) or hexane–ethyl ether (2 to 1). The developed plates were scanned in a thin-layer plate scanner to detect radioactive spots. The radioactive areas were scraped into a scintillation cocktail, and counted in the scintillation spectrometer. The percentages found were calculated according to the quantity applied to the thin-layer plate.

Coincident with the spotting of extracts, known compounds were added and cochromatographed. These known materials were detected by spraying with a ferric chloride–ferricyanide reagent (Abdel-Wahab *et al.*, 1966). Additional confirmation of the chromatographic identity of radioactive materials was produced by exposure of the thin-layer plates to x-ray film, followed by chromogenic detection of cochromatographed known compounds. Identity was established when the radioactive spots revealed by x-ray film and the thin-layer plate scanner coincided exactly with the chromogenically detected spots.

RESULTS AND DISCUSSION

Approximately 75% of the carbonyl-labeled Zectran fed to mice resulted in $^{14}CO_2$ (Table I), indicating that hydrolysis of the carbamate was the major route of breakdown (Krishna and Casida, 1966). Acetylated Zectran (carbamyl carbonyl)-^{14}C, also produced approximately a 75% yield of $^{14}CO_2$, indicating hydrolysis. The feeding of the (acetyl carbonyl)-^{14}C-*N*-acetyl Zectran to mice resulted in approximately 65% of the radioactivity in the urine. Less than 3% $^{14}CO_2$ was found with the use of (dimethylamino)-^{14}C-labeled acetylated Zectran, indicating that the removal of the $^{14}CH_3$ was not the main route for detoxification.

Water-soluble metabolites of Zectran found in dog urine (Williams *et al.*, 1964) were hydrolyzed by strong acid (HCl) to produce the precursor aglycone, 4-dimethylamino-3,5-xylenol.

Table II. Total Recovery of Radioactivity from Spruce Budworm Treated with *N*-Acetylated Zectran

Chemical and Position of ^{14}C Label	Hours	Per Cent Recovery CO_2	Vials	External	Internal	Total
Carbamyl carbonyl-^{14}C-*N*-acetyl Zectran	2	8.9	10.0	60.3	15.7	93.9
	4	24.1	...	...	...	...
	24	43.5	...	...	...	...
	30	54.0	...	...	...	...
	48	61.7	14.0	3.1	16.8	95.6
Dimethylamino-^{14}C-*N*-acetyl Zectran	4	0.9	49.4	34.6	13.8	98.7

Table III. Distribution and Recovery of Radioactive *N*-Acetylated Zectran and Metabolites Resulting from Thin-Layer Chromatography of Spruce Budworm Extracts

Chemical	Sample	Per Cent ^{14}C Recovery *N*-acetyl Zectran	Zectran	Zectran (M.A.)	Total % recovery
Carbamyl carbonyl-^{14}C-*N*-acetyl Zectran	Vials	20.8	23.1	2.3	46.2
	External	17.8	12.6	1.1	31.5
	Internal	3.0	6.2	0.6	9.8
					87.5
Dimethylamino-^{14}C-*N*-acetyl Zectran	Vials	27.0	16.7	...	43.7
	External	20.6	14.3	...	34.9
	Internal	2.1	4.8	...	6.9
					85.5

Using the same technique, we found that the water-soluble radioactive materials in the urine of mice fed (dimethylamino)-^{14}C-*N*-acetyl Zectran produced 67 to 71% of the same aglycone, indicating hydrolysis of the starting *N*-acetylated Zectran.

It is postulated that the major metabolite found in mouse urine as a result of feeding acetyl-labeled Zectran was methylacetamide. We could not absolutely identify this water-soluble metabolite because of the interferences of large quantities of acetamide normally present in mouse urine. The lack of a suitable chromogenic detecting agent also prevented identification.

The use of trap II did not result in the trapping of any radioactive compounds, eliminating the possibility of organic fragments from the radiolabeled *N*-acetyl Zectran, such as methane-^{14}C, or acetic acid-^{14}C. It also showed that evaporation of the starting material was not a factor.

In spruce budworm, $^{14}CO_2$ appeared at a slower rate as a function of the breakdown of the acetylated Zectran (Table II). The major compounds extracted from the vials and from washed and homogenized insects were Zectran, *N*-acetyl Zectran, and 4-methylamino-3,5-xylyl-*N*-methylcarbamate (Zectran, M.A.) (Table III). The thin-layer chromatographic

Table IV. R_f Values[a] of *N*-Acetylated Zectran Metabolites

Origin of Sample	*N*-Acetyl Zectran	4-Dimethylamino-3,5-xylenol	Zectran	Zectran (M.A.)	Solvent[b] System
Mouse urine	...	0.56	...	...	A
	...	0.42	...	...	B
	...	0.32	...	...	C
Insects	0.67	...	0.47	0.17	A
Controls	0.70	0.57	0.47	0.17	A
	0.55	0.46	0.26	0.11	B
	0.45	0.33	0.15	0.10	C

[a] Average of 12 or more thin-layer plates. [b] Solvent system A. Ether–hexane 4:1. B. Ether–hexane 2:1. C. Hexane–ether 2:1.

methods previously described were used for identification. The R_f values are shown in Table IV. Very little $^{14}CO_2$ was found as a result of treating budworm with the (dimethylamino)-$^{14}CH_3$-*N*-acetyl Zectran. Roberts *et al.* (1969) also found this to be true with this label on Zectran, indicating that removal of this group is not a major route of detoxification. Roberts *et al.* did not show that $^{14}CO_2$ was a major metabolite with (carbamyl carbonyl)-^{14}C-Zectran. In our study, addition of an *N*-acetyl group to Zectran showed extensive hydrolysis (Table II).

The acetylation of Zectran did not greatly alter the desired toxic effects on spruce budworm because deacetylation occurred before hydrolysis, producing the toxic precursor Zectran. The toxicity to the mouse was greatly reduced because the animal detoxified the acetylated carbamate by hydrolysis, producing $^{14}CO_2$ when the carbamyl carbonyl was radiolabeled. The use of the 4-dimethylamino label showed the presence of a major metabolite that was a water-soluble conjugate of 4-dimethylamino-3,5-xylenol.

LITERATURE CITED

Abdel-Wahab, A. M., Kuhr, R. J., Casida, J. E., J. AGR. FOOD CHEM. **14,** 290 (1966).
Fraser, J., Clinch, G. P., Reay, R. C., *Sci. Food Agr.* **16,** 615 (1965).
Hayes, F. N., Schrodt, A. G., Gibbs, J. A., Ninth Symposium on Advances in Tracer Methodology, San Francisco, 1964.
Jeffay, H., Alvarez, J., *Anal. Chem.* **33,** 612 (1961).
Krishna, J. G., Casida, J. E., J. AGR. FOOD CHEM. **14,** 98 (1966).
Look, M., J. AGR. FOOD CHEM. **16,** 893 (1968).
Metcalf, R. L., Osnian, M. F., Fukuto, T. R., *J. Econ. Entomol.* **60,** 445 (1967).
Miskus, R. P., Look, M., Andrews, T. L., Lyon, R. L., J. AGR. FOOD CHEM. **16,** 605 (1968).
Pieper, G. R., Miskus, R. P., J. AGR. FOOD CHEM. **15,** 915 (1967).
Roberts, R. B., Miskus, R. P., Duckles, C. K., Sakai, T. T., J. AGR. FOOD CHEM. **17,** 107 (1969).
Robertson, W. A. H., Fraser, J., Clinck, G. C. (to Bootes Pure Drug Co.), Brit. Patent **982,235** (Feb. 3, 1965).
Smith, G. N., Ludwig, P. D., Wright, K. C., Bauriedel, W. R., J. AGR. FOOD CHEM. **12,** 172 (1964).
Williams, E., Meikle, R. W., Redemann, C. T., J. AGR. FOOD CHEM. **12,** 457 (1964).

Received for review July 19, 1968. Resubmitted January 27, 1969. Accepted March 6, 1969.